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Role of CD40 in Prion Disease and the Immune Response to Recombinant PrP

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Abstract

The CD40 receptor-CD40 ligand (CD40-CD40L) interaction has been shown to affect both immune and non-immune cells and is implicated in diverse activities including immunoglobulin class switching (IgM to IgG), atherosclerosis, chronic inflammation and Alzheimer's Disease pathogenesis. A number of groups have studied the role of CD40 in prion disease, however, the results are conflicting presumably due to the use of different scrapie agent-host strain combinations and routes of infection. In the current study, we clarify the effect of CD40 on: (i) replication, progression to clinical disease, PrP^{Sc} profile, and neuropathology associated with infection of a single host genotype with three distinct mouse-adapted scrapie strains, and (ii) the immune response of double knockout (PrP, CD40) transgenic mice to recombinant PrP as assessed by the generation of anti-PrP antibodies. Our results suggest that CD40: (i) results in slower disease progression and scrapie strain-specific differences in incubation periods, (ii) does not affect the level of scrapie strain-specific PrP^{Sc}, (iii) does not influence disease-associated neuropathology, but (iv), as expected, is required to mount an immune response generating anti-PrP IgG antibodies.

Keywords

CD40; prion disease; PrP knockout mice; CD40 knockout mice; recombinant PrP; adjuvants; anti-PrP antibodies; T cell-dependent antigen

Introduction

CD40 receptor (CD40) is a member of the tumor necrosis factor receptor (TNFR) superfamily, which also includes TNFR I and II, and the low affinity p75 nerve growth factor (NGF) receptor (Grimaldi et al., 1992; Torres and Clark, 1992). The interaction of CD40 with its ligand (CD40L) has been shown to have a major effect on cellular and humoral immune responses. CD40 promotes priming of T-helper cells, and interaction

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between T-cell CD40L and B-cell CD40 results in B-cell proliferation and differentiation into antibody-secreting plasma cells (Xu et al., 1994; Foy et al., 1996). Microglial cells express CD40 at low levels and this expression is markedly increased by proinflammatory cytokines such as IFN- γ (Carson et al., 1998; Nguyen et al., 1998; Tan et al., 1999b). Additionally, ligation of microglial CD40 leads to microglial activation, TNF α production and neuronal injury (Tan et al., 1999b). Mice deficient for CD40 or CD40L have impaired immunoglobulin class switching, lack germinal center formation and are deficient for germinal center B cells (GCBs) (Kawabe et al., 1994; Xu et al., 1994). The expression of CD40 by a wide range of cell types, including B lymphocytes, monocytes/macrophages, endothelial cells, smooth muscle cells and cultured human myoblasts, neurons and neuron-like cells (Mach et al., 1997; Brouty-Boye et al., 2000; Sugiura et al., 2000; van Kooten, 2000; Tan et al., 2002b) suggests that it may be multifunctional and play a role that extends beyond its influence on the immune system. Tan et al. (2002b) reported that CD40 influences neuronal development, maintenance and survival *in vitro* and *in vivo*. They also showed that CD40-deficient mice demonstrated age-dependent neurodegenerative changes suggesting that CD40-deficient neurons are more vulnerable to stress associated with aging. In addition, CD40 protected cultivated neurons against the adverse effects of nerve growth factor- β (NGF- β) and serum deprivation (Tan et al., 2002b).

The CD40-CD40L interaction has also been implicated in the pathogenesis of Alzheimer's Disease (AD) and has been proposed as a target for therapeutic intervention (Calingasan et al., 2002; Tan et al., 2002a, c). Evidence from *in vitro* experiments suggested a role for CD40-CD40L in the activation of microglia by β -amyloid (A β) (Tan et al., 1999a, c). Transgenic mice that overproduce A β peptide, but are deficient for CD40L, exhibited decreased gliosis and reduced A β plaque loads compared to mice with unimpaired CD40L signaling. The authors suggest that the reduction in CD40L-activated microglia might oppose plaque pathology, and also demonstrated a role for CD40L in amyloid precursor protein processing and brain-to-blood clearance of A β (Tan et al., 2002a, c). Thus, a therapeutic value of disrupting CD40-CD40L signaling on A β plaque loads needs to be balanced against loss of function important for neuronal survival.

There are conflicting reports regarding the role of CD40 in prion diseases. Deficiency of CD40L has been reported to be detrimental in prion diseases and suggested a neuroprotective function of intact CD40-CD40L interactions. Burwinkel et al. (2004) reported that intracerebral (ic) infection of CD40L-deficient (CD40L^{-/-}) mice with the 139A mouse-adapted scrapie strain resulted in a shorter incubation period than wild-type (WT) control mice and exhibited more extensive neuropathology in the neuropil and increased microglia activation. Further, Mabbott et al. (2003) showed that CD40^{-/-} mice infected via skin scarification with the mouse-adapted ME7 scrapie strain exhibit extensive clinical disease. In contrast, results obtained from CD40L^{-/-} mice inoculated via an intraperitoneal (ip) route with the Rocky Mountain Laboratory (RML) scrapie strain failed to show a protective role for CD40L in prion pathogenesis (Heikenwalder et al., 2007). It is possible that the use of various inocula (RML vs. 139A vs. ME7), in combination with different mouse strains (C57BL/6 vs. C57BL/6 x 129Sv) and different routes of infection (ip vs. ic vs. scarification) is the source of the discrepancies between these studies.

To clarify the role of CD40 in prion disease, we used three mouse-adapted scrapie strains, each with their own unique biological and PrP-associated physiochemical properties to determine whether the CD40 receptor affects host infection, agent replication, and progression to clinical disease. Additionally, in a parallel study we analyzed the influence of CD40 on the immune response of single knockout (PrP^{-/-}) and double knockout (PrP^{-/-} : CD40^{-/-}) mice when challenged with species-specific recombinant PrP immunogens.

Materials and Methods

Mouse Strains

Wild-type FVB/N [WT(FBN)] and Balb/cJ [WT(Balb/c)] mice were obtained commercially from Jackson Labs (Bar Harbor, ME). The PrP knockout mice (PrP^{-/-}) (Bueler et al. 1992) were originally a kind gift from Dr. Charles Weissman and, following backcrossing on an FVB background for more than 10 generations with monitoring by polymerase chain reaction (PCR), maintained by us for many years as homozygous PrP knockout mice (PrP^{-/-}-FVB) by brother-sister mating. The CD40 knockout mice (CD40^{-/-}) were originally generated by Kawabe et al (1994), and obtained commercially (CNCr.129P2-*Cd40^{tm1Kik}/J*, Jackson Labs) on the Balb/c background. We maintained these mice on both the Balb/c background (CD40^{-/-}-Balb/c) by brother-sister mating for infectivity studies and also backcrossed these mice onto the FVB background for a minimum of 10 generations with continual monitoring by PCR (see below). Following the backcrossing to yield a stable FVB genotype, brother-sister mating and PCR monitoring was used to generate the homozygous CD40^{-/-} mouse line (CD40^{-/-}-FVB).

The genes that code for murine PrP and CD40 are both located on chromosome 2 approximately 20 centimorgans apart. The double knockout transgenic mouse line, PrP^{-/-} : CD40^{-/-}, was generated by continually mating homozygous PrP^{-/-}-FVB and CD40^{-/-}-FVB mice and using PCR to select male and female offspring in which the necessary crossing-over events occurred to produce individual mice in which both PrP and CD40 genes were absent. These mice were used to generate breeding pairs and to establish the PrP^{-/-} : CD40^{-/-}-FVB mouse line (referred to in this manuscript as PrP^{-/-} : CD40^{-/-}) which was used for assessment of PrP antibody production.

Scrapie strains, animal infections

Mouse-adapted scrapie strains ME7 and 22L were originally provided by Dr. Alan G. Dickinson and the mouse-adapted 139A scrapie strain by Richard H. Kimberlin (both formerly of the ARC and MRC Neuropathogenesis Unit, Edinburgh, Scotland). Groups of 8–10 WT(Balb/c) mice and CD40^{-/-}-Balb/c mice were injected ic with each of the three mouse-adapted scrapie strains. The preparation of the inoculum, injection, scoring, and sacrificing of animals were performed as previously described (Kascsak et al., 1986).

Western Blotting

Ten percent brain homogenates were prepared in ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, pH 8.0) and centrifuged at low speed (2,000 xg for 10 min). Ten µl of the supernatants were mixed with a final of 1x sample buffer (6 mM Tris-HCl, 1% SDS, 1% 2-mercaptoethanol, 5% glycerol, and 0.01% bromophenol blue, pH 6.8), heated at 100° C for 4 min and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (constant voltage of 100 V) using 12% acrylamide gels. The resolved proteins were electroblotted to nitrocellulose membranes (40 V for 18 hrs) and the membranes were incubated in blocking buffer (3% BSA in PBS) for 1 hr at room temperature. The blots were washed in PBST and incubated with Mab 11F12 (1:5,000 in PBST) for 1 hr at 37° C. Following PBST washes, the blots were incubated with secondary antibody (1:2,000 in PBST) [either goat anti-mouse IgG (Fab fragments) conjugated to alkaline phosphatase with NBT and BCIP as the substrate or horseradish peroxidase-conjugated to goat anti-mouse IgG with super signal west femto maximum sensitivity substrate (Pierce) as previously described (Chang et al., 2008)] for 1 hr at room temperature. For samples that were PK digested prior to SDS-PAGE, 40 µl of the supernatants from the low speed centrifugation were incubated with 100 µg/ml PK (final concentration) for 30 min at 50° C followed by the addition of 1% PMSF, 1X SDS-PAGE sample buffer and heating at 100° C for 5 min.

Histological Analysis

Animals were euthanized using isoflurane inhalation and brains were collected for histological analysis. Brains were fixed for a minimum of 48 hrs by immersion in 4% paraformaldehyde, dehydrated in increasing concentrations of ethanol, cleared in xylene, and embedded in paraffin. Eight-micrometer-thick, serial coronal sections were cut using a rotary microtome. Brain sections were stained with hematoxylin-eosin (H-E) and the extent of pathological changes (spongiosis, neuronal loss, astrogliosis) in the cortex and hippocampus was quantified stereologically by a rater blinded to the experimental status of the mice. The analysis was performed on ten consecutive cross-section profiles separated by 600 μ m using a systematic, random sampling scheme free of operator-driven bias. Differences in quantified measures of brain pathology were analyzed using one-way ANOVA followed by a Tukey-HSD post-hoc test. The statistical and power analyses were performed using Graph Pad Prism Software v4.03 (Graph Pad Prism Software, Inc., San Diego, CA, USA) and CSS Statistica v6 (StatSoft, Inc. Tulsa, OK).

Immunogens, Adjuvants and Immunizations

The full-length recombinant PrP (rPrP) from mouse (m-rPrP), two sheep genotypes: sheep (A₁₃₆R₁₅₄R₁₇₁) (shpARR-rPrP) and sheep V₁₃₆R₁₅₄Q₁₇₁ (shpVRQ-rPrP), hamster (ha-rPrP), and deer (d-rPrP) were generous gifts from Drs. David Brown and Paul Davies (University of Bath, UK) and described previously (Chang et al., 2009). The human A β ₁₋₄₂ (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA) was generated commercially (Sigma Chemical Co.). The three adjuvants, Freund's (Sigma Chemical Co.), Inject Alum (Thermo Scientific), and Titermax Gold (CytRx, Norcross, GA) were compared. Fifty μ g of immunogen (0.1 μ g/ μ l) per subcutaneous (sc) injection per mouse was used to immunize the PrP^{-/-}-FVB, PrP^{-/-} : CD40^{-/-}, and the WT(FVB) mice. The immunogens were prepared with Freund's or Titermax Gold at 1:1 ratios as per manufacturer's instructions. With Freund's, an initial immunization was performed using immunogen combined with Freund's complete adjuvant (FCA) followed by three subsequent boosts of immunogen with Freund's incomplete adjuvant (FIA). Alum was mixed 1:1 with the immunogen for 30 min at room temperature before injection. For all the adjuvant-immunogen combinations a minimum of 3 mice were used. Each mouse received a total of 4 immunizations with a 3–6 week interval between injections. Mice were bled on day 6 after the 4th injection and the sera was monitored by ELISA for an immune response.

ELISA

ELISAs were performed in triplicate using high binding 96-well plates that were coated at 4° C overnight with immunogen (100 ng/well) diluted in carbonate buffer, pH 9.2. The plates were blocked for 2 hrs at 37° C with casein in TBS supplemented with 0.1% Tween 20 and 0.05% sodium azide. Serial dilutions of sera were evaluated for their antibody response to the specific antigens. After a 2 hr incubation at 37° C and 3 consecutive washes in TBS-0.1% Tween 20 (TBST), a 1:5,000 of the secondary antibody [alkaline phosphatase-conjugated goat anti-mouse IgG (Fab specific) or goat anti-mouse IgM (u-chain specific) (Sigma)] was added. After another 2 hr incubation at 37° C and 3 consecutive TBST washes, the complex was detected by development with 1mg/ml 4-nitrophenyl phosphate disodium salt hexahydrate dissolved in 0.01% MgCl₂, 0.3 M HCl, and 10% diethanolamine. After incubation for 1hr at 37° C, absorbance readings were measured at 405 nm.

PCR

Mouse genomic DNA is extracted by treatment of ear punch or 3 mm tail tissue with 50 mM NaOH at 98° C for 1 hr. After cooling, add 1/10 volume of 1M Tris-HCl, pH 8.0, centrifuge at 13,200 $\times$ g for 3 min and collect the supernatant.

For PCR analysis of the murine PrP gene, primers P3 (5'-ATTCGACGCGCATCGCCTTCTATCGCC-3'), P4 (5'-CCTGGGAATGAACAAAGGTTTGCTTTCAAC-3'), and P10 (5'-GTACCCATAATCAGTGAACAAGCCCAGC-3') (Bueler et al., 1992) were used together. For CD40, primers Tnfr IMR692 (5'-GGCAGTAAGACGATGTGACAACAGAG-3') and Tnfr IMR157 (5'-TCGTCCTGCAGTTCATTTCAG-3'), and Tnfr IMR694 (5'-GAGATGAGAAGGAAGAATGGGAAAAC-3') were used.

The PCR reactions contained (in a final volume of 25 μ l): 0.2 mM dNTPs, 0.48 μ M P3 and P10 (or IMR694 and IMR157), 0.96 μ M P4 (or IMR692), 1.5 mM MgCl₂, 1x GoTaq Flexi PCR reaction buffer, 1 Unit GoTaq Flexi DNA polymerase (Promega), and 2 μ l of 1:10 diluted mouse genomic DNA. PCR consisted of 1 cycle at 94° C for 3 min followed by 35 cycles at 94° C for 90 sec, 65° C for 2 min, and 72° C for 2 min, and then a final incubation of 72° C for 10 min. The PCR products were electrophoresed on 1% agarose gels in TAE buffer at 5 V/cm followed by ethidium bromide staining. The expected results are as follows: (i) For analysis of PrP with the P3 / P4 / P10 primer group, PrP^{-/-}-FVB mice have an amplified PCR product of 0.85 kb (P3 / P4) while WT(FVB) and CD40^{-/-}-FVB mice have a 1.1 kb amplified PCR product (P4 / P10) and, (ii) For analysis of CD40 with the IMR157 / IMR692 / IMR694 primer group, WT(FVB) mice and PrP^{-/-}-FVB mice have an amplified PCR product of 0.89 kb (IMR692 / IMR694) while CD40^{-/-}-FVB mice have a 1.2 kb amplified PCR product (IMR692 / IMR157). Therefore, the PrP^{-/-} : CD40^{-/-} double knockout transgenic mice are expected to have only PCR products of 0.85 kb (P3 / P4) and 1.2 kb (IMR692 / IMR157) (Fig. 5).

Results

The role of the CD40/CD40L interaction during a prion infection of the CNS, and the question of whether ablation of the CD40 gene would affect agent replication, was assessed by comparing the replication and disease progression of the three mouse-adapted scrapie strains 139A, ME7, and 22L following ic infection of WT(Balb/c) and CD40^{-/-}-Balb/c mice. Survival curves of CD40^{-/-}-Balb/c and WT(Balb/c) mice (Fig. 1) indicates that in the two mouse lines infected with each of the scrapie strains, overall, clinical signs initially appeared earlier in the WT(Balb/c) mice but disease progression is more rapid in the CD40^{-/-}-Balb/c mice. The differences in disease progression between WT(Balb/c) and CD40^{-/-}-Balb/c was most pronounced for the 139A scrapie strain followed by ME7 and was least discernible for 22L. Although the CD40 deficiency leads to more rapid disease progression, it did not modify the clinical symptoms displayed by the infected animals, which included somnolence, kyphosis, tremors, stilted gait, and ataxia. The mean incubation periods for each group of mice were compared (Table 1). There were no significant differences in the mean incubation periods between CD40^{-/-} mice and WT mice infected with the 139A scrapie strain (146+1 vs. 156+13). Similar conclusions were made for the ME7 infected mice (156+5 for CD40^{-/-} vs. 156+12 for WT mice) and for the 22L-infected CD40^{-/-} mice compared to the WT (148+4 vs. 154+4). We also observed scrapie stain-specific effects on the incubation periods. Interestingly, all three scrapie strains replicated equally well in the WT(Balb/c) mice but showed differences in incubation periods in the CD40^{-/-} mice with ME7 having the longest and most closely comparable to WT(Balb/c) mice while 139A had the shortest (Table 1).

To further characterize the scrapie-infected CD40^{-/-}-Balb/c and WT(Balb/c) mice, we examined the accumulation of the disease-associated abnormal isoform of the prion protein, PrP^{Sc}. Brains from clinical CD40^{-/-} and WT mice following infection with 139A, ME7 and 22L scrapie strains were harvested, homogenized and PK treated prior to western blotting

and immunostaining with the PrP-specific Mab 11F12. PK studies demonstrated that, as expected, PrP^C was PK sensitive showing complete degradation and that the PK resistance of PrP^{Sc} was similar between the two mouse lines for any given scrapie strain (Fig. 2). Furthermore, for the individual scrapie strains, the banding pattern of the PK-treated PrP^{Sc} was indistinguishable when infected CD40^{-/-} mice were compared to WT mice (Fig. 2). The PrP^{Sc} banding pattern profiles showed scrapie strain specificity and was characteristic for each of the scrapie agents as previously reported (Kascsak et al., 1986). PK-resistant PrP^{Sc} from the brains of ME7 and 22L infected CD40^{-/-} and WT mice consisted of three equally immunoreactive bands in the 20–30 kDa region while the PrP^{Sc} banding pattern from 139A-infected CD40^{-/-} and WT mice also showed three bands with the most slowly migrating species displaying less immunoreactivity than the two lower ones (Fig. 2).

We compared the neuropathology present in 139A, ME7, and 22L scrapie strain-infected WT(Balb/c) and CD40^{-/-}-Balb/c mice at the terminal stages of clinical disease. We evaluated the degree of spongiosis, neuronal loss, and astrocytosis in the cortex and hippocampus. For each of the three scrapie strains, no significant difference in the pathology was found when the brain regions of WT(Balb/c) mice were compared to CD40^{-/-}-Balb/c mice. In addition, the pathology in the brains from clinical WT(Balb/c) were similar to each other regardless of the scrapie strain used. This was also the case when the brain pathology among the CD40^{-/-}-Balb/c mice infected with the various scrapie strains was compared (Figs. 3 and 4).

The high homology of mammalian PrP^C between species makes a host tolerant to the antigenic PrP^{Sc} or recombinant PrP and thus it is difficult to initiate an immune reaction (Rubenstein et al., 1999). Therefore, to generate a strong immune response to PrP, it has been a standard practice to use PrP^{-/-} mice as a host to generate anti-PrP antibodies. These mice do not express the normal cellular PrP isoform (PrP^C) and are, therefore, not tolerant of the PrP antigen. We examined the influence of CD40 on the ability of mice to generate an immune response to PrP by establishing a double knockout transgenic mouse line, PrP^{-/-} : CD40^{-/-} on an FVB genotype background. Analysis of the amplified products following PCR of DNA from homozygous PrP^{-/-} : CD40^{-/-} mice and comparison between the expected (see Materials and Methods) and observed sizes of the amplified PCR products demonstrate that these mice lack both the PrP and the CD40 genes (Fig. 5) and therefore lack PrP and CD40 expression.

The immune response of PrP^{-/-} : CD40^{-/-}, PrP^{-/-}-FVB, and WT(FVB) mice were compared following immunization with a variety of antigens (rPrP, A β , BSA) using several adjuvants. The PrP^{-/-}-FVB, PrP^{-/-} : CD40^{-/-} and WT(FVB) mice were injected with full-length ha-rPrP, d-rPrP, m-rPrP, shpARR-rPrP, and shpVRQ-rPrP using Freund's adjuvant following the immunization and sample collection timetable described in Materials and Methods. The ELISA results (Table 2) demonstrated that the injected PrP^{-/-} mice generated the strongest IgG immune response to all rPrP immunogens. Not surprisingly, the WT mice also generated a fairly good IgG response (although not as strong as the PrP^{-/-} mice) to all rPrP except, as expected, m-rPrP. We anticipated that any detectable IgM immune response would not be substantial with our immunization and sera testing protocol due to the time span between Ig class switching and sera collection. As expected, although the PrP^{-/-} : CD40^{-/-} mice did not generate a IgG immune response, we were able to detect a low level IgM immune response, to all of the rPrP immunogens. Since there was no detectable IgG and the generation of IgM is an initial immune response, it is probable that a significant IgM concentration would only be present early in the immunization process.

We next sought to determine whether the lack of immune responses observed in PrP^{-/-} : CD40^{-/-} mice were specific only to the prion protein or was it a more generalize

phenomenon. Human A β ₁₋₄₂ and bovine serum albumin (BSA) were used as additional immunogens for PrP^{-/-}-FVB, PrP^{-/-} : CD40^{-/-}, and WT(FVB) mice. The degree of IgG and IgM immune responsiveness for A β ₁₋₄₂ and BSA measured in all three mouse lines was similar to that observed for rPrP (with the exception of m-rPrP in WT mice) with PrP^{-/-} mice showing the strongest IgG response followed by WT mice and no detectable IgG from PrP^{-/-} : CD40^{-/-} mouse sera. We next sought to determine whether the immune responsiveness of the mice was influenced by the adjuvant used. In addition to the results described above using Freund's, we also examined the ability of mice to generate an immune response using two additional adjuvants, Alum and Titermax, in combination with m-rPrP and shpARR-rPrP (Table 2). The immunization and sample collection timetable was the same one used for Freund's. The IgG and IgM immune responses detected with Alum and Titermax were similar to that for Freund's. PrP^{-/-} mice gave the strongest IgG response to both immunogens (with low levels of IgM) while the WT mice responded well with an IgG response to shpARR-rPrP and not to m-rPrP. Once again, low levels of IgM and no IgG was detected in PrP^{-/-} : CD40^{-/-} mouse sera.

Discussion

With the development of CD40^{-/-} and CD40L^{-/-} mice, it was demonstrated (Kawabe et al., 1994; MacDonald et al., 2002; Tumanov et al., 2003) that these mice: (i) are able to elicit normal IgM and IgG responses to thymus-independent antigens, (ii) generate IgM responses but no IgG, IgA, and IgE responses to thymus-dependent antigens, and (iii) demonstrate defective germinal center formation with intact primary B-cell follicles. These results indicate that CD40, CD40L, and their interaction are necessary for T cell-dependent Ig class switching and GCB formation, but not for T cell-dependent IgM responses and T cell-independent antibody responses.

Although previous reports (Tan et al., 2002a, b, c; Laporte et al. 2006) indicated that CD40 signaling is required for the expression of complete of AD-like pathology, our studies suggest that CD40 does not play a similar role in prion disease pathology. The actual role of CD40 and the influence it has on different cell types with respect to the scrapie infection process is still unclear. Conflicting reports regarding the influence of CD40 on scrapie strain replication, the progression of clinical disease and neuropathogenesis has resulted in uncertainty over its role in prion diseases. Heikenwalder and colleagues (2007) reported that neuropathogenesis associated with peripheral prion disease infection with the Rocky Mountain Laboratory scrapie strain was not influenced by the presence or absence of CD40L. They reported that the onset of clinical signs, disease duration, and histopathological features, as well as levels of PrP^{Sc} from clinically-affected scrapie brains of mice which do not express CD40L, were similar to CD40L-expressing control mice. The authors concluded that GCBs alone do not impact peripheral splenic prion transport, replication efficiency, or neuroinvasion, and point to other mechanisms affecting prion transport from lymphoreticular sites of replication to the nervous system. In contrast, studies by Burwinkel et al. (2004) using CD40L^{-/-} mice infected ic with the 139A scrapie strain reported that a deficiency of CD40L exacerbates prion diseases and that the function of intact CD40-CD40L interactions are to provide a neuroprotective role. Furthermore, Tan et al (2002b) reported extensive neurodegeneration in aging CD40^{-/-} mice. Our findings both support and contrast those of previous studies (Burdwinkel et al., 2004; Mohan et al., 2005; Heikenwalder et al., 2007). Mohan et al. (2005) reported that WT mice and CD40L^{-/-} mice challenged ic with the ME7 scrapie strain developed clinical signs of scrapie with similar incubation periods and CNS pathology. This is in agreement with our results with the ME7 scrapie strain. Based on histopathological evaluation of clinical mouse brains after infection with three distinct scrapie strains, our studies do not support a pronounced neuroprotective role for CD40, as reported by Burwinkel et al. (2004), since the neuropathology was

indistinguishable between normal and CD40 deficient mice. However, with respect to incubation periods, the presence of CD40 only lengthened the course of the 139A infection thereby demonstrating a degree of neuroprotection with this agent but not with ME7 or 22L. Furthermore, we not only observed a more rapid onset of clinical signs but also a slower disease progression in CD40-containing mice. A possible explanation for our findings is that CD40 might have separate roles related first to infection and second to replication of the agents. The presence of CD40 may initially function as an ancillary cell receptor for the infectious agents such that its absence would lessen the amount of agent actually binding to the cells and thus lengthen the beginning of the infection process. However, once the binding has taken place and infection process continues, the role of CD40 might be to provide an intra-and/or intercellular environment that limits the rate of agent replication.

Alternatively, following the binding event, agent replication may be affected by a CD40-CD40L signaling mechanism. Mohan and others (2005) showed that CD40L^{-/-} mice, infected with the ME7 scrapie strain via skin scarification, developed disease earlier than WT mice. They hypothesized that Langerhans cells residing in the epidermis play an influential role in scrapie pathogenesis through a CD40L-dependent mechanism. In addition, the CD40-CD40L interaction leads to microglial and macrophage activation (Tan et al., 1999a; 2002a, c). Although immunocompetent microglia cells are involved in the pathogenesis of prion disease, their role in the disease process is still unclear (Rezaie et al., 2001). Microglia have been shown not only to destroy scrapie-infected neurons *in vitro*, but culture media from PrP^{Sc} activated microglia can induce neurotoxicity in normal cultures (Combs et al., 1999). It is therefore possible that activated microglia kill infected neurons and, during this process, reduce the levels of, and essentially inactivate, the scrapie agent. Therefore, it is possible that the increased rate of disease progression in CD40^{-/-} mice is related to the lack of activated microglia.

Our observations on agent replication do not support the concept that considers CD40-CD40L interactions as neuroprotective since absence of CD40-CD40L signaling does not alter the extent of neuropathology or the levels of PrP^{Sc} in infected mice for all three scrapie strains. However, it is unknown, although possible, for CD40L to bind to receptors other than CD40 with an effect that may compensate for the CD40-CD40L interaction. Other ligands of CD40, namely C4b-binding protein (C4BP) and the 70 kDa heat shock protein (hsp70) could functionally replace CD40L (Becker et al, 2002; Brodeur et al, 2003).

Our initial goal was to take advantage of the role of CD40 in immune responses and immunoglobulin class switching to generate anti-PrP IgM antibodies. We developed and immunized the PrP^{-/-} : CD40^{-/-} mouse line in order to generate a limited immune response and the production of IgM-specific anti-PrP antibodies. These mice generated a low level immune response not only to PrP, but also A β and BSA. The inability to generate an IgG response indicates that PrP antigen processing is a T cell-dependent event. This IgM response was similar even when measured 1 week after immunization (data not shown). It is possible, though unlikely, that the IgM response was influenced by the adjuvant. However, the animals' responses to the antigens were similar regardless of the adjuvant used. Adjuvants play an important role in establishing an immune response to an exogenously introduced antigen. We examined several adjuvants whose modes of action differ from one another. However, the inability of the PrP^{-/-} : CD40^{-/-} to generate an immune response was independent of the adjuvant used. Adjuvants enhance the humoral and/or cell-mediated immune response to antigens by assuring proper antigen presentation to the antigen-presenting cells and macrophages which, in turn, secrete cytokines that influences Ig isotypes, isotype switching and further macrophage activation. Activated macrophages secrete a variety of proinflammatory cytokines. TH1 cells secrete IL2 and INF- γ which activate macrophages and cell-mediated immunity while TH2 cells secrete mainly IL4,

IL10, and IL13 which influence isotype switching in the humoral immune response as well as depressing macrophage activation. Aluminum adjuvants exhibit some immunostimulatory properties and they promote an almost exclusive TH2 response to the antigen whereas Freund's and TiterMax induce mainly a TH1 response.

The expression of CD40 on numerous different cell types implies multiple roles. It has been shown that the CD40-CD40L interaction can maintain immune homeostasis and be both immunostimulatory and immunosuppressive. The strong influence that CD40 has on immune regulation is exemplified in our studies on the response, or lack of, in mice to PrP immunogens. Additionally, this single receptor-ligand pair can be responsible for a reciprocal principle not only of immune regulation (Mathur et al., 2006), but as we now demonstrate, can also be extended to other biological processes such as disease promotion and disease preventing.

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Figure 1.

Survival curves of CD40^{-/-}-Balb/c and WT(Balb/c) mice following infection with the 139A, ME7, and 22L mouse-adapted scrapie strains. Mice were infected, monitored for clinical disease, and incubation periods determined as described in Materials and Methods.

Figure 2.

Western blot analysis of PK-resistant PrP^{Sc} from the brains of WT(FVB) mice (two mice per scrapie strain) and symptomatic CD40^{-/-}-FVB mice (two mice per scrapie strain) infected with the 139A, ME7, and 22L mouse-adapted scrapie strains. Brain homogenates, PK treatment and western blot analysis was performed as described in Materials and Methods. Untreated and PK-treated brain homogenate from uninfected WT(FVB) mouse was also western blotted to demonstrate complete PrP^C digestion by PK.

Figure 3.
Histological analysis of cortex from symptomatic WT(Balb/c) (A–C) and CD40^{-/-}-Balb/c (D–F) mice infected with the 139A (A, D), ME7 (B, E), and 22L (C, F) mouse-adapted scrapie strains. Scale bar is 50 microns

Figure 4. Histological analysis of hippocampus from symptomatic WT(Balb/c) (A–C) and CD40^{-/-} Balb/c (D–F) mice infected with the 139A (A, D), ME7 (B, E), and 22L (C, F) mouse-adapted scrapie strains. Scale bar is 50 microns

	PCR Product (kb)	
	<u>P3 / P4 / P10</u>	<u>IMR692 / 694 / 157</u>
Lanes 1 and 5 – WT(FVB) mouse	1.1	0.89
Lanes 2 and 6 – PrP ^{-/-} -FVB mouse	0.85	0.89
Lanes 3 and 7 – CD40 ^{-/-} -FVB mouse	1.1	1.2
Lanes 4 and 8 – PrP ^{-/-} : CD40 ^{-/-} mouse	0.85	1.2

Figure 5.

Analysis of amplified PCR products using DNA from WT and gene knockout mouse lines. Details of the PrP primer set (P3 / P4 / P10) and CD40 primer set (IMR 692 / 694 / 157) are in Materials and Methods. There is a good correlation between the expected (see Materials and Methods) and observed sizes of the amplified PCR products.

Table 1Incubation Periods of Scrapie Strain-Infected CD40^{-/-}-Balb/c and WT(Balb/c) Mice.

<u>Mouse Line</u>	<u>Scrapie Strain</u>		
	<u>139A</u>	<u>ME7</u>	<u>22L</u>
CD40 ^{-/-} -Balb/c	146 ± 1 *	156 ± 5	148 ± 4
WT(Balb/c)	156 ± 13	156 ± 12	154 ± 4

* Incubation period (days post-infection) expressed as mean ± standard deviation

Table 2

Comparison of Immune Responses from PrP^{-/-}-FVB, PrP^{-/-} : CD40^{-/-}, and WT(FVB) Mice.

Adjuvant	Immunogen	ELISA^a		
		PrP^{-/-}	PrP^{-/-} : CD40^{-/-}	WT
Freunds	ha-rPrP	>3.0 : 1.8 ^b	0 : 0.6	1.8 : 0.7
	d-rPrP	>3.0 : 0.4	0 : 0.8	2.1 : 0.7
	m-rPrP	>3.0 : 0.7	0 : 0.4	0 : 0
	shpARR-rPrP	>3.0 : 0.3	0 : 0.8	2.2 : 1.0
	shpVRQ-rPrP	>3.0 : 0.5	0 : 0.4	1.9 : 0.9
	human Aβ 1-42 ^c	>3.0 : 0.9	0 : 0.4	2.8 : 1.1
	BSA ^d	>3.0 : 1.3	0 : 0.7	>3.0 : 1.2
Alum	m-rPrP	>3.0 : 0.4	0 : 0.4	0 : 0
	shpARR-rPrP	>3.0 : 0.5	0 : 0.5	2.2 : 0.6
Titer Max	m-rPrP	>3.0 : 1.9	0 : 0.8	0 : 0
	shpARR-rPrP	>3.0 : 0.5	0 : 0.7	2.6 : 0.9

^aData is shown using sera dilutions of 1:5000 and 1:100 for IgG and IgM determinations, respectively. ELISA readings are expressed as immunogen-specific OD405 with a maximum possible recordable OD405 of 3.0.

^bIgG : IgM

^cDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA

^dBovine Serum Albumin